The Immunology of Hantavirus and Helminth Co-infections in Bank Voles

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Tiivistelmä - Referat – Abstract

Co-infection, a state in which the host is infected with more than one micro- or macroparasite at a time, is the norm in the wild because of a wide range of interacting organisms and parasites. Bank vole is a reservoir host of Puumala hantavirus (PUUV), a pathogen causing Nephropathia Endemica, an endemic disease in Finland. The helper T cell (Th)1/Th2 polarization theory, which is established in the laboratory, but less-studied in the wild, suggests that there is a trade-off between Th1 response against microparasites and Th2 response against macroparasites. I studied whether helminth or hantavirus infection, individually and synergistically, have effect on the immune responses of wild bank voles and whether there is a trade-off between Th1 and Th2 responses. My hypothesis was that helminth infection would reduce the bank voles' ability to mount an effective immune response against viral infections and make them more susceptible to chronic Puumala virus infection. I measured mRNA levels of transcription factors *Tbet* (Th1 response) and *Gata3* (Th2 response) in the splenocytes of wild-caught bank voles after stimulating the cells with different immune stimulants. I also measured the constitutive levels of *Tbet* and *Gata3* in bank voles' spleens. The splenocytes of PUUV-infected bank voles were less responsive to stimulations than those of PUUV-negative ones. The reduced ability of splenocytes from PUUV-infected voles to respond to stimulation can be because of the virus itself affecting the T cell function or alternatively due to an inherent defect in immune cells making them more susceptible to PUUV infection. The constitutive expression of *Gata3* in spleen correlated positively with gastrointestinal nematode load in PUUV-infected voles but not in PUUV-negative voles. This can be because of mounting an immune response against helminths reduces the bank voles' ability to resist the viral infection in accordance with the trade-off between Th1 and Th2 responses or as previous studies have shown, *Gata3* can act as a marker of infection tolerance in bank voles. Because of a small sample size and a heterologous group of studied bank voles, more research is needed on co-infection immunology in bank voles and other wild animals.

Luonnossa eläimet altistuvat jatkuvasti monille erilaisille taudinaiheuttajille, kuten viruksille, bakteereille ja loisille. Tämän takia yhteisinfektio on luonnossa ennemmin sääntö kuin poikkeus. Yhteisinfektio tarkoittaa useamman kuin yhden taudinaiheuttajan aiheuttamaa samanaikaista infektiota. Metsämyyrä on hantaviruksiin kuuluvan Puumala-viruksen (PUUV) isäntälaji, ja viruksen aiheuttama myyräkuume on Suomessa endeeminen infektiotauti. Teoria auttaja-T-solujen (Th-solu) aiheuttamien Th1-ja Th2-soluvasteiden polarisaatiosta on todistettu laboratoriotutkimuksissa, mutta siitä ei ole vielä kovin paljon näyttöä luonnossa. Teorian mukaan solunsisäisiä patogeenejä, kuten viruksia ja bakteereja, vastaan syntyvät Th1-soluvasteen ja loisia vastaan syntyvän Th2-soluvasteen vastaan välillä vallitsee polarisoiva tasapainotila, jossa toisen vasteen aktivoituessa kyky toisen immuunivasteen tuottamiseen heikkenee. Tutkin hantavirus- ja suolistoloisinfektioiden vaikutusta, sekä yhdessä että erikseen, villien metsämyyrien immuunivasteisiin ja Th1- ja Th2-soluvasteiden välistä polarisaatiota. Hypoteesini oli, että suolistoloisinfektio vähentää metsämyyrien kykyä tuottaa tehokas Th1-soluvaste virusinfektiota vastaan ja tekee ne alttiimmaksi krooniselle Puumala-virusinfektiolle. Mittasin kahden transkriptiotekijän, Th1-soluvasteen *Tbet*:n ja Th2-soluvasteen *Gata3*:n lähetti-RNA:n ilmentymistä PCR-menetelmällä metsämyyrien pernasoluissa erilaisilla immunostimulanteilla aiheutetun stimulaation jälkeen. Mittasin myös *Tbet*:n ja *Gata3*:n fysiologisia ilmentymistasoja metsämyyrien pernoissa. PUUV-infektoituneiden myyrien pernasolut stimuloituivat heikommin kuin myyrien, joilla ei ollut Puumala-virusinfektiota. Tämä saattaa johtua joko siitä, että Puumala-virus vaikuttaa solujen toimintaan jollakin tavalla tai vaihtoehtoisesti myyrien immuunisolujen toimintahäiriön takia niillä on suurempi alttius saada Puumala-virusinfektio. *Gata3*:n fysiologinen ilmentymistaso pernassa korreloi positiivisesti suolistossa olevien pyörömatojen lukumäärän kanssa PUUV-infektoituneilla myyrillä, muttei niillä, joilla ei ollut PUUV-infektiota. Tämä saattaa johtua joko siitä, että immuunivaste suolistoloisia vastaan heikentää myyrien kykyä vastustaa virusinfektiota Th1- ja Th2-soluvasteiden polarisaation takia. *Gata3* voi myös toimia infektiotoleranssin markkerina metsämyyrillä. Melko pienen näytemäärän ja tutkittavien eläinten monien eroavaisuuksien vuoksi lisää tutkimuksia aiheesta tarvitaan sekä tämän työn tulosten tueksi, että ymmärtämään yhteisinfektioiden vaikutusta immuunivasteisiin metsämyyrillä ja muilla villieläimillä.

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1 INTRODUCTION

Living in the wild exposes an individual to a wide range of parasites, including micro- and macroparasites (Jackson et al. 2009). That makes a co-infection, a state in which an organism is infected by multiple parasites at the same time, almost a basic state for natural populations (Cox 2001, Telfer et al. 2010). In a natural setting with limited resources, there are also other environmental stressors to deal with, so an individual needs to make trade-offs with immune responses fighting off parasites and other life history traits, such as breeding, to ensure their survival (Sheldon and Verhulst 1996).

The immune response can involve trade-offs between pathways. The classical example is the antagonism between the T helper (Th) cell 1 mediated Th1 response against microparasites, such as bacteria and viruses, and Th2 response against macroparasites, like helminths (Kidd 2003). Naïve T cells differentiate to different T cell subsets based on the cytokines present in the activation process (Wan and Flavell 2009, Zhu et al. 2010). Naïve T cell can only differentiate to one type of Th cell, and so in theory different T cell pathways can antagonize each other so that strong upregulation of Th2 response can lead to reduced capacity to mount a strong Th1 response and vice versa (Openshaw et al. 1995, Romagnani 1996, Zhu et al. 2010). This means that an animal infected with helminths should be more susceptible to virus infections and have a reduced capacity to control them after the infection (Hellard et al. 2015, Vaumourin et al. 2015), but while the theory of polarized Th1/Th2 responses has been established in the laboratory, it hasn't been widely recognized in the wild.

Although classical immunological research has provided a lot of information on the mechanistic basis of the immune system, only little is known about its function in natural settings (Pedersen and Babayan 2011). Furthermore, most research on host-parasite interactions has focused on single infections and to understand natural systems we need to understand the consequences of co-infections for the host and host populations (Lehmer et al. 2018). Wild rodents are known reservoirs of a range of zoonotic pathogens (pathogens, that can transmit between animals and humans), such as Puumala virus, so the knowledge about host-parasite interactions of wild rodents can help understand and minimize the impact of zoonotic pathogens on human health (Meyer and Schmaljohn 2000, Bordes et al. 2015).

In this thesis, I study the immune responses of bank voles (*Myodes glareolus*) against Puumala virus and intestinal helminths both individually and synergistically. Firstly, in order to measure the ability of PUUV-infection to affect bank vole immunity, I stimulated bank voles splenocytes with different T cell stimulants, such as proinflammatory stimulants lipopolysaccharide (LPS), imiquimod and zymosan and general stimulants phytohaemaglutinin (PHA) and phorbol myristate acetate (PMA) with ionomycin and after that, measured transcription factor (*Tbet* for Th1 pathway and *Gata3* for Th2 pathway) mRNA levels with quantitative PCR to assess the type and intensity of Th cell response. In addition, since the use of the above-mentioned stimulants are poorly described for wild animals such as bank voles, I investigated the general responsiveness of bank vole splenocytes to the applied stimulation. Secondly, I measured the constitutive levels of *Tbet* and *Gata3* expression in spleen in a physiological state to assess the ratio between the two in relation to PUUV and helminth infection status.

Overall, my aim is to answer questions like: do PUUV-infected bank voles have a reduced ability to mount a Th1 response? If so, is that because of helminth infection? Is there a trade-off between Th1 and Th2 responses, so that when other is stronger, the other is weaker and vice versa? Is there a difference between Th1 and Th2 response expression in a physiological state, and if there is, is it a consequence of Puumala virus or helminth infection? My hypothesis is that intestinal helminths will induce a Th2 response which reduces the bank vole's ability to produce an effective Th1 response against Puumala virus and makes them more susceptible to chronic Puumala virus infection.

2 LITERATURE REVIEW

2.1 Eco-immunology

2.1.1 Introduction to eco-immunology

This thesis studies co-infections of bank voles using an eco-immunological approach. Ecological immunology (eco-immunology) is an emerging interdisciplinary research field, and its main goal is to reach a better understanding of the causes and consequences of variation in immunological processes in natural populations and their ecological and evolutionary impact (Sheldon and Verhulst 1996, Sadd and Schmid-Hempel 2009, Downs et al. 2014). Eco-immunology combines knowledge and techniques from both laboratory and field research from many scientific disciplines, such as classical immunology, disease ecology, neuroendocrinology and genomics (Pedersen and Babayan 2011, Downs et al. 2014). Eco-immunology investigates how biotic and abiotic factors interact with immune system in an individual and how those consequences affect the population dynamics of hosts and parasites and between individuals and species (Martin et al. 2011, Downs et al. 2014).

Immunological processes have been extensively studied, but most of the knowledge comes from research done in controlled laboratory setting, mostly with inbred laboratory mice (Sadd and Schmid-Hempel 2009, Pedersen and Babayan 2011). Although very important, this knowledge doesn't always translate well to natural settings because of the large amount of variation in individuals and environment they live in (Pedersen and Babayan 2011).

One of the key discoveries of eco-immunology is that the function of immune system is costly and needs resources, which are usually limited in natural settings (Sheldon and Verhulst 1996, Martin et al. 2006a). With limited resources, the investment into immune function is a trade-off with other physiological functions (Sheldon and Verhulst 1996, Sadd and Schmid-Hempel 2009). These trade-offs have been studied extensively for many life-history traits and across multiple species (Downs et al. 2014). Other important concept of eco-immunology and disease ecology is a dichotomy of the host response to a pathogen: resistance and tolerance (Downs et al. 2014). Resistance means the ability to fight the parasite and reduce its number and tolerance means minimizing the harm caused to host fitness during the infection (Downs

et al. 2014). These two different host responses alter the evolution of host-pathogen systems and disease prevalence in complex and often different ways (Downs et al. 2014).

2.1.2 Eco-immunology tools

A major challenge of eco-immunology has been to find suitable techniques, both technical and statistical, to use in wild, non-model systems, and developing and refining methods has been a key challenge for the research field (Pedersen and Babayan 2011, Downs et al. 2014). Many of the techniques used in eco-immunology still rely on traditional immunological laboratory methods, but sophisticated laboratory technology has become affordable during the last decade, which has opened many doors for eco-immunologists (Pedersen and Babayan 2011).

Some of the traditional assays used to measure immunity in wildlife are bacterial killing assays (BKA), haematological analyses including white blood cell count (WBC) and neutrophil: leukocyte (N:L) ratio, or H:L (heterophil: leukocyte) ratio in birds, delayed type hypersensitivity (DTH) assays and immunoglobulin (Ig) antibody levels (Demas et al. 2011, Strandin et al. 2018). There are many more methods that have been used in measuring immune responses in wild animals as well, as reviewed by Demas et al. (2011).

Genomic and post-genomic technologies, which have become more affordable lately, have paved their way into eco-immunological research (Pedersen and Babayan 2011, Downs et al. 2014). New sequencing technologies have made it possible to map significant parts of the genome of almost any species, and from the knowledge of *Mus musculus* genome, it has been possible to identify genes, including immune genes, of other rodents (Pedersen and Babayan 2011). With new gene mapping technology, the quantification of many cytokines, immunological markers and chemokines has become possible, which has led to a better understanding of immune phenotypes (Downs et al. 2014). Technologies and methods, such as peripheral-blood transcriptome, new ELISA technologies and identification and quantification of serum microRNAs are some additional examples of methods, that will shed new light on eco-immunological research in the future (Pedersen and Babayan 2011, Downs et al. 2014).

2.2 Co-infections

2.2.1 Co-infections and immunology

Co-infection, also known as multiparasitism, is a state in which the host is infected by multiple parasites, including microparasites (e.g. viruses and bacteria) and macroparasites (e.g. helminths), concurrently (Fenton et al. 2008, Vaumourin et al. 2015). Most infections of animals, especially wild animals, but also humans, are coinfections, and this has been recognized in research only recently (Bordes and Morand 2011, Vaumourin et al. 2015). Research on multiparasitism is important to understanding natural infection processes because parasites interact in many ways and can alter host responses to other parasites (Pedersen and Fenton 2007, Vaumourin et al. 2015).

Parasite interactions within a host are either direct, when they affect each other's survival by interference competition, or indirect when they interact via host immune response (Hellard et al. 2015). The interactions of parasites can be synergistic, which means that they can facilitate other parasite's infections or antagonistic, when parasites inhibit consequent infections by other parasites (Hellard et al. 2015, Vaumourin et al. 2015). Parasites can influence the host response so that other parasite can infect the host easier or can alter the duration of infection, symptoms or transmission of other parasites (Vaumourin et al. 2015).

Parasites need to gain entry to a host to infect it and they do it primarily mechanically (Bandilla et al. 2006, Vaumourin et al. 2015). Parasites can facilitate other parasites' entrance to hosts, such as gaining entry from lesions in mucous membranes and skin caused by other parasites (Bandilla et al. 2006, Vaumourin et al. 2015). For example, rainbow trout with ectoparasites have been shown to be more susceptible to bacterial infections (Bandilla et al. 2006), and Herpes virus infection have been demonstrated to facilitate HIV infection in humans (Van de Perre et al. 2008). Other forms of interactions between parasites occur, for example, through interference by producing toxins that kill other parasites or by competing from the same space (Pedersen and Fenton 2007, Hellard et al. 2015). Competition of same available resources and space, exchange of genes and alteration of another parasites gene expression are also ways in which parasites can interact (Hellard et al. 2015).

Parasites often interact with host's immune system and its immune memory (Cox 2001, Vaumourin et al. 2015). They can alter the host's immune system and responses in ways that suppress it and thus facilitate other infections, and they can also enhance it and inhibit other infections (Hellard et al. 2015, Vaumourin et al. 2015). Parasites can cause an immunosuppression or it can alter the activation state of immune cells and thus facilitate concurrent infections by suppressing host immune responses (Hellard et al. 2015). An example of enhancing the host's immune system and inhibiting other parasites is cross-immunity, where antibodies against one parasite can work on other antigenically similar parasites as well (Hellard et al. 2015, Vaumourin et al. 2015).

An important mechanism of parasites influencing the host immune response and concurrent infections is a trade-off between Th1 and Th2 responses (Cox 2001, Vaumourin et al. 2015), although the theory still needs more research in natural populations. Th1 and Th2 responses are polarized and antagonize each other (Kidd 2003). The Th1/Th2 trade-off causes dynamic interactions between parasites, as one response suppresses the other, so an infection inducting the other response reduces the host's immune response to a parasite inducing the other (Hellard et al. 2015, Vaumourin et al. 2015). The classic example of this trade-off is a coinfection of helminths (Th2 response) and microparasites (Th1 response), which has been studied in humans and animals, also in wild animals (Pedersen and Fenton 2007, Ezenwa 2016). In humans, helminth infection has been shown to increase the susceptibility to malarial infections (Nacher et al. 2002). Helminth (nematode *Heligmosomum mixtum*) co-infection has also been shown to increase the susceptibility to Puumala virus infection in bank voles (Salvador et al. 2011, Guivier et al. 2014). Co-infections are quite extensively studied, but there is not that much research on immunological mechanisms behind the micro- and macroparasite co-infections in natural populations.

2.2.2 Th1 and Th2 pathways

CD4+ T lymphocytes are an important part of the immune system: they mediate adaptive immunity against many types of pathogens and play a role in some diseases (Zhu et al. 2010). Naïve CD4+ T cells differentiate into different types of T helper cells (Th cells) after activation: Th1, Th2, Th17, Th9, Th22, regulatory T (T_{reg}) cells and T follicular helper (T_{fh}) cells (Schmitt and Ueno 2015). Th cells are classified by the cytokine profile they produce,

except for T_{reg} cells and T_{fh} cells, which are defined by their function, location, and transcription factors and markers they express (Schmitt and Ueno 2015). Th cells have a major role in the immune system functions: they help and regulate other immune cells, such as B cells, CD8+ cytotoxic T cells and macrophages, control immune responses and are a part of immunological memory (Zhu et al. 2010).

The Th1/Th2 pathway theory is originally based on the study from 1986, where Mosmann et al. (1986) distuingished two subtypes of mouse-derived Th cells by the cytokines they secreted and their functions: Th1 and Th2 cells (Kidd 2003). Based on the new information about other subsets of T helper cells and immune cell functions, the Th1/Th2 pathway theory is a bit oversimplified, but still holds true in most cases and is a good basis for understanding immune functions (Romagnani 1996, Zhu et al. 2010). Because a precursor cell can differentiate to one type of Th cell, Th1 and Th2 pathways are polarized and antagonize each other at least to some extent (Openshaw et al. 1995, Romagnani 1996, Zhu et al. 2010).

The Th1 pathway is responsible for cell-mediated immunity and for immune responses against intracellular pathogens including many bacteria, viruses and protozoal parasites (Kidd 2003, Wan and Flavell 2009). Th1 cells also play a role in defending against cancer cells (Micallef et al. 1997) and are responsible for delayed type hypersensitivity skin reaction, which is usually a reaction against bacterial and viral antigens (Kidd 2003, Wan and Flavell 2009). The pro-inflammatory properties of Th1 pathway activation can also cause tissue damage and are considered responsible for autoimmune diseases such as type-1 diabetes and rheumatoid arthritis (Kidd 2003, Wan and Flavell 2009). The main cytokine produced by Th1 cells is interferon-gamma (IFN-γ), and others are for example lymphotoxin, tumor necrosis factor (TNF)- β, TNF-α and IL-12 (Wan and Flavell 2009, Zhu et al. 2010). Th1 cells activate macrophages, cell cytotoxicity dependent on antibodies and promote the production of opsonizing antibodies by B cells and thus, cause phagocyte-dependent inflammation response (Romagnani 2000).

Th2 pathway defends hosts against extracellular pathogens such as helminths, and is considered a part of humoral immunity (Kidd 2003, Wan and Flavell 2009). Th2 pathway activation is also linked to allergies and atopies and other IgE based hypersensitivities (Kidd 2003, Wan and Flavell 2009). Th2 pathway cytokines promote antibody production, IgG1 and IgE class switching, and activate eosinophils, mast cells and inhibit the function of phagocytic

cells (Romagnani 2000, Wan and Flavell 2009). Th2 cells produce interleukine-4 (IL-4), and IL-5, IL-9, IL-10 and IL-13 (Wan and Flavell 2009).

Naïve, undifferentiated CD4 T cells are activated through T cell receptor (TCR)-mediated process (Zhu et al. 2010). Dendritic cells, macrophages and monocytes and other antigen-presenting cells (APCs) recognize pathogen antigens with pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) (Kidd 2003, Akira et al. 2006). Antigen-presenting cells interact with naïve T cells by presenting antigens and secreting cytokines and thus activating the polarization process (Kidd 2003, Akira et al. 2006). They become Th0 cells at first, which is a transient, short phase before differentiating to functional Th cells (Kidd 2003). The transcription factors expressed and the genes transcribed are the main determinants for the differentiated Th cell subsets (Zhu et al. 2010). The main determinant for the Th cell type, to which the naïve T cells differentiate, is considered to be the cytokines present in the TCR activation process (Wan and Flavell 2009, Zhu et al. 2010). The most important cytokines that promote Th1 differentiation are IFN-γ and IL-12 and IL-4 for Th2 differentiation (Wan and Flavell 2009). The main transcription factors to promote Th1 differentiation are *Tbet, Hlx* and *STAT4*, and for Th2 differentiation *Gata3, STAT6* and *c-Maf* (Wan and Flavell 2009). Measuring the cytokines and transcription factors of different immunological pathways can then give information on what kind of response is upregulated during studied infections (Downs et al. 2014).

2.2.3 Bank voles as the model system for co-infections

There are many advantages of using rodents as a model system for studying immune responses and co-infections in the wild (Bradley and Jackson 2008). Reagents used in immunological laboratory methodology are mainly designed for model organisms, most often a mouse (*Mus musculus*), and humans (Bradley and Jackson 2008). The studied species should therefore be closely related to model organisms, as for example antibodies and genome sequences designed for mice and rats usually work for wild rodent species too (Bradley and Jackson 2008). Using wild rodents as a model system can also give information on whether the knowledge of co-infection immunology and immune responses obtained from model organisms in laboratory can be applied to natural populations (Behnke et al. 2001). It is good to choose model systems that can be used alongside laboratory infection models, because the

data from field studies is more challenging to interpret (Bradley and Jackson 2008). Rodents are known to carry many zoonotic pathogens and with the ongoing species extinction, rodents can likely be the dominant mammals in human-modified environments in the future and that makes studies on rodents and pathogens important for human health too (Bordes et al. 2015). Some practical advantages of using bank voles as the model system are that they are abundant in Finnish forests (Stoltz et al. 2011) and are relatively easy to catch and handle. Diagnostic tools are available for the pathogens of bank voles (for example Stoltz et al. 2011) as they are quite well characterized (for example Tadin et al. 2012).

Bank voles are known carriers of two microbes supposedly associated with either Th1 or Th2 immunity; Puumala virus and helminths, respectively. Puumala virus causes a persistent and mainly asymptomatic infection in its natural reservoir host species, a bank vole (*Myodes glareolus)* (Vaheri et al. 2013a). Puumala virus is a causative agent of Nephropathia epidemica (NE), an endemic disease in Finland, and the prevalence of NE in humans is linked to abundance of bank voles with fluctuating population cycles (Vaheri et al. 2013a, Sane et al. 2016). Helminths are known to have immunomodulatory effects on their hosts and they can suppress the immune system (Maizels and Yazdanbakhsh 2003, Jackson et al. 2009), and with cytokines of Th2 pathway, helminth infection can antagonize an effective Th1 response against microparasites, such as Puumala virus and make the hosts more susceptible to concomitant infections (Salgame et al. 2013). Co-infection with a nematode *Heligmosomum mixtum* has been linked to higher PUUV viral load in bank voles in a study by Salvador et al. (2011). In a study by Grzybek et al. (2015), *H. mixtum* was one of the most common nematodes of the bank vole with a prevalence of up to 80 percent, almost the same as the prevalence of helminth infection.

2.3 Puumala virus

2.3.1 Zoonotic hantaviruses

Puumala virus belongs to the *Orthohantavirus* family (hereafter referred to as Hantaviruses), in the order *Bunyavirales* (Klempa 2018). Hantaviruses are enveloped RNA viruses (Jonsson et al. 2010, Vaheri et al. 2013b). The molecular analysis of Hantaan virus (HTNV) was first done and described by Schmaljohn and Dalrymple (1983) and it showed that the genome of

hantavirus is comprised of three negative-sense, single-stranded RNAs (Jonsson et al. 2010). The three segments are called small (S), medium (M) and large (L) and they share a 3' terminal sequence (Schmaljohn and Dalrymple 1983). The genome is the size of about 12 000 nucleotides (Jonsson et al. 2010) and it encodes nucleocapsid protein, glycoprotein precursor and viral RNA-depended RNA-polymerase and sometimes a reading frame for a non-structural protein, that may act as an interferon inhibitor (Vaheri et al. 2013b). They have a diameter of 120 to 160 millimeters and are round or pleiomorphic and have an enveloped virion which is covered in spikes (Huiskonen et al. 2010, Hepojoki et al. 2012). The spiky structure of hantaviruses is thought to be unique amongst enveloped viruses (Huiskonen et al. 2010) Hantaviruses are stable, and can survive and remain infectious around 12-15 days in room temperature and more than 18 days at 4 $^{\circ}$C and at – 20 $^{\circ}$C but are inactivated at 37 $^{\circ}$C after 24 hours (Kallio et al. 2006a).

Hantaviruses are viruses hosted mainly by rodents, but also shrews, bats and moles, and found in Europe, Asia, Africa and Americas (Jonsson et al. 2010). Hantaviruses usually have one or few reservoir host species and are distributed geographically according to reservoir host species (Vaheri et al. 2013b). Hantaviruses cause mainly asymptomatic, persistent infection in their reservoir host (Vaheri et al. 2013a). Some hantaviruses are zoonotic, and can cause severe, even fatal disease in humans (Jonsson et al. 2010). Hantaviruses are divided to Old World and New World hantaviruses based on the geographic distribution of the reservoir species and the disease (HFRS and HPS) they cause to humans (Jonsson et al. 2010). Examples of Old World hantaviruses are Hantaan virus (HTNV) in Asia, Dobrava virus (DOBV) and Puumala virus (PUUV) in Europe and Seoul virus (SEOV) across the World (Jonsson et al. 2010, Vaheri et al. 2013b). New World hantaviruses are for example Sin nombre virus in North America and Andes virus (ANDV) in South America (Vaheri et al. 2013b). HFRS is caused by Old World hantaviruses and HPS by New World hantaviruses (Jonsson et al. 2010).

New hantavirus species have been identified in shrews, moles and bats in a growing rate (Holmes and Zhang 2015), and recently from insects as well (Shi et al. 2016). However, at present, all the zoonotic hantaviruses are rodent-borne, and there is no evidence of hantaviruses of bats, moles and shrews to cause disease in humans (Holmes and Zhang 2015). Hantaviruses have traditionally thought to have coevolved quite strictly with their one rodent

or insectivore host species (Plyusnin et al. 1996), but recent information on host species diversity host jumping and local host-specific adaptation has shown, that their evolutionary history is more complex than previously thought (Yanagihara et al. 2014, Holmes and Zhang 2015).

2.3.2 Hantavirus diseases in humans

Humans get hantavirus infection most often by inhaling rodent excreta containing the virus (Vaheri et al. 2013b), but human-to-human transmission is also reported from South American Andes virus (ANDV) (Martinez et al. 2005). Hantaviruses cause two diseases in humans: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS), also known as hantavirus cardiopulmonary syndrome (HCPS) (Jonsson et al. 2010, Avšic-Županc et al. 2015). HFRS is reported worldwide, especially in Europe and Asia, and HCPS is caused by hantaviruses in North and South America (Vaheri et al. 2013a). The milder form of HFRS is called Nephropatia Endemica (NE) and is caused by Puumala virus (PUUV) (Vaheri et al. 2013a). Mortality rates for HFRS are up to 10 %, depending on the virus, while HPS is usually higher at around 25-35 % (Krüger et al. 2011). The mortality rate of NE is significantly lower at around 0.1-0.4 % (Krüger et al. 2011).

HFRS and HPS are acute, generalized infections, and they can affect many organs: kidneys, heart, lungs and the central nervous system (Vapalahti et al. 2003, Vaheri et al. 2013b). Typical symptoms of HFRS are high fever, nausea and vomiting and abdominal and back pain (Vaheri et al. 2013b, Avšic-Županc et al. 2015). In addition, visual disturbances and somnolence are also commonly reported (Vapalahti et al. 2003, Avšic-Županc et al. 2015). As the disease progresses, hemorrhages, abdominal and pleural effusion, hypotension (sometimes leading to shock) and acute kidney injury may be seen (Vaheri et al. 2013a). HPS is a disease with a clinical course ranging from mild to severe that can lead to rapid progression of respiratory failure leading to cardiogenic shock (Jonsson et al. 2010, Avšic-Županc et al. 2015). The initial symptoms are very similar with HFRS, with additionally myalgia, cough, diarrhea and chills are reported often (Vaheri et al. 2013b). Increased capillary permeability and acute thrombocytopenia are important characteristics of hantavirus diseases and many of the observed symptoms can be explained through these two phenomena (Vaheri et al. 2013b, Avšic-Županc et al. 2015).

Diagnosis is based on clinical symptoms, anamnesis and laboratory tests (Avšic-Županc et al. 2015). The definitive diagnosis is based on serology because virtually all patients have antibodies in serum once the symptoms have started (Jonsson et al. 2010). As there is no specific antiviral therapy generally used for HFRS and HPS in Europe or the Americas, respectively, treatment for hantavirus diseases is mainly supportive (Jonsson et al. 2010, Avšic-Županc et al. 2015). Although, an antiviral drug ribavirin has been shown to have some efficiency for treating HFRS if given early enough in course of the disease (Huggins et al. 1991), but it has not been shown effective for treatment of HPS in clinical trials (Jonsson et al. 2010).

Prevention of hantavirus is based on protective measures and rodent control (Vaheri et al. 2013a, Avšic-Županc et al. 2015). Limitation of food sources and shelter opportunities from rodents near houses and elimination of rodents from human dwellings and avoiding contaminated areas and using protective measures are the basic measures of prevention (Avšic-Županc et al. 2015). Some inactivated hantavirus vaccines are available in Asia, and several others are in pre-clinical development stages, but none have been approved for the European market (Krüger et al. 2011, Avšic-Županc et al. 2015).

2.3.3 Puumala virus infections in Finland

Nephropathia endemica (NE), a mild form of HFRS caused by Puumala virus (PUUV), is endemic in northern Europe (Vaheri et al. 2013a, Sane et al. 2016). Hantavirus infection is a disease that needs to be reported to public health authorities in many countries in Europe (Vaheri et al. 2013a, Sane et al. 2016). Most HFRS cases in Europe are from Finland, and other countries with cases also common in are Sweden, Belgium, France, Germany and the Balkans (Vaheri et al. 2013a).

The annual incidence of reported PUUV infection cases in Finland between 1995 and 2014 was on average 31 cases/100 000 (Sane et al. 2016). Highest incidence was in Eastern Finland, and the lowest in Southwestern Finland (Sane et al. 2016). Latronico et al. (2018) reported a seroprevalence of 12.5% in Finland. They counted from that seroprevalence that around 446/100 000 person-years would be the estimated incidence rate (Latronico et al.

2018). The difference between reported cases and estimated incidence rate means that most of the infections go undetected, most likely because they are mild and don't need medical care (Latronico et al. 2018).

Most PUUV cases occur in late summer and early winter (Vaheri et al. 2013a, Sane et al. 2016). In urban areas especially in Southern Finland, there is a peak in August, because people have been spending their summer vacation in summer cottages, and get the infection from there (Sane et al. 2016). Other peak is around November and December, which is most likely caused by rodents coming to seek shelter from the early winter in and nearby human housing (Vaheri et al. 2013a, Sane et al. 2016).

There are some seasonal tendencies in PUUV incidence, although the temporal changes in seasonality differ in different regions in Finland (Sane et al. 2016). The regional differences in seasonality are most likely caused by an interaction between climate and bank vole population dynamics (Sane et al. 2016). Some of the differences could possibly also be explained by the regional differences in human activities and the use of protective measures (Sane et al. 2016). Human PUUV infection rates follow the fluctuation cycles of bank vole populations (discussed further) with a few months' lag (Sane et al. 2016).

There are some risk factors that have been linked to PUUV infection. Infection prevalence has been shown to be higher in farmers and they get the infection earlier than others in same age group (Vapalahti et al. 1999). There are more reported cases in males, 61 %, which could be because of their activity habits (e.g. more time spent outdoors) or because infection is milder in females (Sane et al. 2016). The highest seroprevalence was in Eastern Finland and in older people, which is explained so that they have had more time of exposure (Latronico et al. 2018). Smoking is also proven to be a risk factor of infection (Vapalahti et al. 2010), and was seen in Latronico's et al (2018) study as well. Other known risk factors for NE are living in a house with holes in it (Vapalahti et al. 2010) and living near the forest (Gherasim et al. 2015). Activities and occupations linked to a higher risk of NE are for example making house repairs, seeing rodents and cleaning their droppings, mowing the lawn and cleaning a summer cottage (Gherasim et al. 2015).

2.4 Bank voles

2.4.1 Bank vole ecology in Finland

Bank voles (*Myodes glareolus*) are distributed from British Isles to most of continental Europe and across Siberia (Hutterer et al. 2016). They inhabit all types of forests and woodlands, and can also be found in hedge networks and vegetated clearings, parks and river banks (Hutterer et al. 2016).

Bank voles don't hibernate during winters, and they are nocturnal in spring and autumn, and diurnal during winter, in summer they are active during night and day (Ylönen 1988). Bank voles have a versatile and mixed diet, which consists of different plant materials, seeds and invertebrates for example (Butet and Delettre 2011).

Bank voles in Finland breed during the summer time, from May to September (Koivula et al. 2003). Females usually give birth to up to four litters in one breeding season, with each litter of 2 to 10 pups (Koivula et al. 2003). Females are territorial during the breeding season (Koskela et al. 1997). Males have bigger home ranges, which overlap female territories (Koskela et al. 1997). During winter, bank voles have been thought to show less territorial aggression and tolerate each other better, because communal living during cold times is likely to benefit their survival, but a recent study by Johnsen and colleagues had contradictory results (Johnsen et al. 2019).

Bank vole populations fluctuate in 3 to 5 year cycles in Finland and boreal Europe (Hanski et al. 2001). The fluctuation is thought to be caused by specialist predators (Hanski et al. 2001). In temperate zones of Europe, population fluctuation is driven by mast years of deciduous trees (Vaheri et al. 2013a).

2.4.2 PUUV disease ecology in bank voles

Bank voles are the reservoir host species for Puumala virus (PUUV) (Meyer and Schmaljohn 2000). Puumala virus causes persistent infection in bank voles, and it is thought to be

asymptomatic (Meyer and Schmaljohn 2000). Lately it's been shown though, that Puumala virus infection affects overwintering success of bank voles negatively (Kallio et al. 2007).

Bank voles shed hantavirus in their urine, feces and saliva (Hardestam et al. 2008). After the initial infection, shedding and viral load is at its highest after 2 to 4 weeks, and then decreases slowly during following months before the shedding stops or decreases only to happen every now and then (Hardestam et al. 2008). The secretion kinetics is studied in laboratory setting though and recent studies from natural populations have shown, that shedding lasts for a lifetime in a significant part of the population (Voutilainen et al. 2015). Lifelong shedding may be an important for sustaining infection in cyclic vole populations that undergo bottlenecks (Voutilainen et al. 2015).

Hantavirus transmission among voles occurs via inhalation of aerosolized virus in excreta or via biting, grooming and other forms of direct contact (Hardestam et al. 2008, Forbes et al. 2018). During the breeding season, aggressive behavior and biting may be an important transmission route (Escutenaire et al. 2002). Outside the breeding season, grooming and communal nesting is thought to play a more important role in the infection transmission (Escutenaire et al. 2002).

Puumala virus prevalence is density-dependent to some point and higher when fluctuating bank vole population is bigger (Olsson et al. 2002), although this has had contradictory results in other studies (Escutenaire et al. 2002, Kallio et al. 2010). PUUV prevalence is higher in males than females (Bernshtein et al. 1999, Olsson et al. 2002), which is thought to be because of their larger ranges and thus more contacts with conspesifics (Olsson et al. 2002). Outside the peak years in population cycles, older males were shown to be significantly more infected with PUUV than older females, and they may play a key role in sustaining the virus in populations (Escutenaire et al. 2002).

The prevalence is higher in the spring, when the population is consisted mostly of overwintered adults (Voutilainen et al. 2016), although the spread of the virus is most likely higher later, when naïve juveniles are likely to get the infection (Boone et al. 2002). The offspring of an infected female voles are protected from the virus through maternal antibodies, which can protect them for up to 80 days (Kallio et al. 2006b). Kallio et al. (2010) showed, that a high PUUV prevalence early in the start of the breeding season resulted in high

maternal antibody levels and thus delayed the transmission of PUUV in population and lowered the PUUV prevalence next year, even when the vole density increased. Maternal antibodies can so be one of the influencers of the transmission dynamics of PUUV in vole populations (Kallio et al. 2010).

Landscape and habitat affect the PUUV prevalence in complex ways, by affecting the population structure and movement (Khalil et al. 2014). Other species affect negatively to the virus prevalence: the bigger diversity, the lower prevalence, which is thought to be caused by fewer contacts with conspesifics, as the habitat is shared by other species (Khalil et al. 2014).

2.4.3 Immunological mediators of hantavirus infections in reservoir hosts

Reservoir host's immune responses to hantaviruses differ from dead-end hosts, such as humans, and is characterized by a persistent infection with a subtle immune response (Easterbrook and Klein 2008). Regulatory T cells (T_{reg} cells) suppress inflammatory T cell responses locally, and thus help pathogen persistence and contribute also to reducing proinflammatory-mediated pathogenesis (Belkaid 2008, Easterbrook and Klein 2008). During persistent hantavirus infection, T_{reg} cell levels are elevated in lungs, a hantavirus replication site (Easterbrook et al. 2007). T_{reg} cells suppress the innate immunity and thus the immune response by suppressing the expression and production of cytokine tumor necrosis factor-alpha (TNF-α) and elevating those of cytokine TGF-β (Easterbrook et al. 2007). T_{reg} cells were associated also to subclinical lung tissue pathology in persistent hantavirus infection (Easterbrook et al. 2007) and may limit the immunopathology in host species (Schountz et al. 2007).

In the lung tissue of hantavirus (SEOV) infected rats, the expression of many antiviral and proinflammatory cytokines and factors, such as IFN-β, IL-1β and IFN-γ, are reduced (Hannah et al. 2008). Also, the expression and function of pattern recognition receptors (PRRs) are decreased, which indicates that virus recognition is inhibited, and can contribute to the infection persistence together with immune response downregulation (Easterbrook and Klein 2008). On the other hand, antiviral and proinflammatory responses are elevated in the spleen during an acute infection and then reduced back to normal, which indicates that hantaviruses

don’t cause an overall immunosuppression rather than a localized reduction in immune responses (Easterbrook and Klein 2008).

Pathogenic hantaviruses, contrary to non-pathogenic hantaviruses, can delay the induction of antiviral immune response, type 1 IFN pathway, and thus mitigate more effective virus replication in human endothelial cells *in vitro* (Kraus et al. 2004, Easterbrook and Klein 2008, Schountz and Prescott 2014). As humans are dead-end hosts for hantaviruses, this mechanism is most likely evolved in reservoir hosts (Schountz and Prescott 2014). In rat lung endothelial cells *in vitro*, after the inoculation of SEOV, virus replication begun and it did not result in cytokine or chemokine production, so the virus was able to inhibit the antiviral response (Li and Klein 2012). The cells also showed an upregulation in T_{reg} cell activity (Li and Klein 2012). Antigen presenting cells (APCs), which are needed for the activation of T cells, were isolated from rats and infected with SEOV *in vitro* (Au et al. 2010). After the infection, APCs’ signaling activity (e.g. production of TNF-α, IL-10 and IL-6 and expression of MHCII) was reduced and normal function inhibited (Au et al. 2010). The inhibition of innate response has been shown in PUUV-infected bank vole cells as well: bank vole embryonic fibroblasts infected with PUUV *in vitro* did not show an upregulation of *Mx2* and IFN-β, which was seen in infection with other viruses (Stoltz et al. 2011).

Some host genetic factors have been associated with PUUV infection in bank voles (Easterbrook and Klein 2008, Vaheri et al. 2013b). Deter et al. (2008) reported an association between PUUV infection and a major histocompatibility complex (MHC) class II gene *DQA* and resistance and susceptibility to PUUV infection. This needs more research though, as seronegative voles could have been resistant to PUUV infection or they haven’t been in contact with the virus (Deter et al. 2008). Polymorphism in a TNF-α promoter gene has been linked to PUUV infection so that the genotype leading to higher levels of TNF-α is more frequent in the areas, where PUUV is not endemic (Guivier et al. 2010). Guivier et al. (2010) also reported that the baseline levels of TNF-α gene expression in bank voles differed so that they were lower in endemic areas than in non-endemic areas. As suppressed TNF-α levels are linked to infection persistence in bank voles, this might suggest, that immune responses and a balance of tolerance and resistance in reservoir hosts can be an important factor of disease ecology of hantaviruses (Guivier et al. 2010).

After infecting with hantavirus SEOV, male rats have more virus in target tissues and shed more virus than females (Hannah et al. 2008). Also factors that contribute to innate antiviral response, like the induction of PRRs and upregulation of the expression of antiviral genes were more efficient in the lungs of female rats (Hannah et al. 2008). After removing the testes from male rats, these differences are evened, which suggests that sex hormones regulate the immune responses (Easterbrook and Klein 2008, Hannah et al. 2008).

2.5 Helminths

2.5.1 About helminths

Helminths are parasitic worms, which infect animals and plants (Taylor et al. 2016). There are many estimations about the number of species in total, one of them being approximately 75 000-300 000 helminth species (Taylor et al. 2016). Helminths of importance to mammal hosts are found in the higher taxa Nematoda (roundworms), Acantocephala (thorny-headed worms) and Platyhelminthes (flatworms), which contain Trematoda (flukes) and Cestoda (tapeworms) (Hofman 2016, Taylor et al. 2016). Helminths are classified by their morphology in all their life stages: egg, larval (juvenile) and adult stage (Hofman 2016).

Helminths can be either hermaphrodites or the sexes can be separate (Taylor et al. 2016). Helminths have distinct life history stages: egg stage, one or multiple larval, juvenile stages and adult stage (Botzler and Brown 2014). Their life cycles can be divided into two categories: direct and indirect (Taylor et al. 2016). In indirect life cycle, they need an intermediate host from other species, and in direct life cycle, helminths infect their definitive host species directly (Taylor et al. 2016). Helminths infect their definitive host in many ways (Botzler and Brown 2014). The most common mechanism is ingestion of an infective stage, usually larval stage or an egg, by definitive host ingesting food or water with infective stages in it or by predating an intermediate host (Botzler and Brown 2014). Other ways of infection are penetration through the skin, which the larva can sometimes do itself or via arthropod vector or transplacental or transmammary transmission (Botzler and Brown 2014).

Nematodes and cestodes were found from the intestines of the studied bank voles. Nematodes, in other name roundworms, are parasitic or free-living helminths (Taylor et al.

2016). They have a cylindrical and unsegmented body, which has a thin, non-cellular outer layer called cuticle (Botzler and Brown 2014, Taylor et al. 2016). Nematodes are an important helminth group with many species causing mortality in wild animals, however free-living nematodes have an important role in food webs as decomposers (Botzler and Brown 2014). Cestodes, in other name tapeworms, have segmented, elongated and flat body as adults (Taylor et al. 2016). The adults inhabit the intestinal lumen or accessory ducts of the definitive host and usually don't cause much adverse effects for the host (Botzler and Brown 2014). The life cycle of most cestodes is indirect and have one or more intermediate hosts (Taylor et al. 2016). In the intermediate or dead-end hosts, larvae parasite other tissues, in cystic or solid forms, which can cause pathology (Botzler and Brown 2014, Taylor et al. 2016).

2.5.2 Disease ecology of helminths and helminth communities in wild rodents

Wild rodents harbor a diverse community of helminths, and most individuals are infected with more than one helminth species concurrently (Haukisalmi and Henttonen 1993, Behnke et al. 2001). As reproduction of helminths inside their hosts doesn't immediately lead to bigger number of parasites and as some of the helminth life cycles occur outside the host, rodent host-helminth population interactions are dynamic (Scott and Lewis 1987). Helminth populations are greatly influenced by the dynamics of their host populations, for example host abundance fluctuations, and can influence host dynamics also by themselves (Scott and Lewis 1987).

The community structure of helminths is shaped by both intrinsic, host-related factors and extrinsic, such as geographical and seasonal, factors (Haukisalmi et al. 1988, Behnke et al. 2001). Although host traits, such as body condition, age, sex and immunocompetence influence the parasite communities, geographical, spatial and temporal factors are shown to be more important determinators (Behnke et al. 2001, Dallas and Presley 2014).

Studies have shown that the helminth community is usually consisted of dominant core helminth species, especially nematodes with quite stable infection patterns and the rare species, which show more random fluctuation (Grzybek et al. 2015). Although many helminth species co-occur frequently in hosts, co-occurence is mostly due to spatial, temporal or other

factors rather than due to interaction between the helminth species, although competition between species occur (Haukisalmi et al. 1988, Behnke et al. 2001). This is contrary to the research done in the laboratory, where synergy between helminth species pairs has been observed (Behnke et al. 2001).

There are pros and cons from helminth species diversity to hosts and host populations. Increased parasite load and helminth species richness have been shown to influence the host fitness by reduced body condition, increased immune investment and more severe infection outcomes (Bordes and Morand 2011, Loxton et al. 2016). At the population level, helminth species richness was shown to be linked to higher polymorphism of MHC class II immune genes, which is indicative of host-parasite coevolution and benefits the host populations (Goüy de Bellocq et al. 2008).

2.5.3 Immunology and pathology of helminth infection

Parasites and hosts often share a long co-evolutionary history (Anthony et al. 2007). Parasites need to survive and reproduce inside the host and to do that, they usually need to interact with the host's immune system (Anthony et al. 2007). At the same time, the host needs to develop an effective immune response to clear the parasite or minimize its harm, but it needs to do it so that it can protect itself against other pathogens as well (Anthony et al. 2007). For these reasons, helminth infections are typically chronic infections with a down-regulated immune response for limiting the immunopathology that might be caused from complete elimination of the parasites (McSorley and Maizels 2012).

As described earlier, macroparasites, such as helminths, induce Th2 immune responses, leading to increased production of IL-4 and other cytokines of Th2 pathway and to activation of eosinophils, mast cells and IgE producing plasma cells (Anthony et al. 2007). Helminth infection also induces a regulatory immune response lead by T_{reg} cells and immunoregulatory cytokine IL-10 (Anthony et al. 2007, McSorley and Maizels 2012). Other cells that play a part in immune response against helminths are for example alternatively activated macrophages (AAMs), regulatory B cells and dendritic cells (McSorley and Maizels 2012). Antibodies and cell types needed for an effective response is dependent on the parasite and its location in the host (Anthony et al. 2007). Helminths have been shown to downregulate the host's immune

response by producing mediators that can affect the function of host's immune system (Maizels and Yazdanbakhsh 2003).

As an example of importance of the immune response and its consequences in helminth infection, effective downregulation of cytotoxic Th1 response has been linked to helminth infections with less symptoms and pathology (McSorley and Maizels 2012). In *Schistosoma* infections, the initial response is Th1 response against the adult parasite, but after it lays eggs, the response changes to Th2 response (Taylor et al. 2016). If the switch between Th1 and Th2 responses doesn't succeed, prolonged Th1 response leads to granulomatous infection and substantial tissue damage with sometimes fatal consequences (Anthony et al. 2007, Taylor et al. 2016).

2.5.4 Helminths of the bank vole

There are several studies investigating the helminth communities of bank voles. The helminth community of Finnish bank voles have been described in a few studies too, although not very recently to my knowledge. The nematode species of Finnish microtine rodents, including bank vole, that were reported in 1983, are *Heligmosomum mixtum, H. yamagutii, H. costellatum, Boreostrongylus minutus, Syphacia stroma, S. nigeriana, S. petrusewiczi, Angiostrongylus dujardini, Capillaria* sp. and *Mastophorus muris* (Tenora et al. 1983). Trematodes reported are *Plagiorchis elegans* and *Notocotylus* sp. and cestodes are *Hydatigera taeniaeformis*, *Taenia tenuicollis*, *Cladotaenia globifera, Anoplocephaloides* sp., *Paranoplocephala* spp. and *Catenotaenia* spp. (Tenora et al. 1983). In a study of Haukisalmi et al. (1988), six helminth species were most common in bank voles, four nematodes and two cestodes. The two most common helminths were *Heligmosomum mixtum* and *Catenotaenia* sp. (Haukisalmi et al. 1988). Other less common helminth species were nematode *Syphacia petrusewiczi, Mastophorus muris,* and *Capillaria* sp and cestode *Paranoplocephala kalelai*. (Haukisalmi et al. 1988). Nematode *Heligmosomoides glareoli* has also been found in Finnish bank voles (Haukisalmi and Henttonen 1993).

In a more recent study conducted in Northeast Poland, the most common helminth species of the bank vole were nematodes *Heligmosomum mixtum*, *Heligmosoides glareoli* and *Mastophorus muris* (Grzybek et al. 2015). In a period of 11 years, their prevalence and

abundance showed stabile patterns in bank vole populations (Grzybek et al. 2015). The overall prevalence of helminth infection was high, around 80 percent and the prevalence of nematode infection was 77 percent (Grzybek et al. 2015). Other species reported from the bank voles in Poland are for example nematode *Aspiculiris tianjinensis, Syphacia petrusewiczi* and *Trichuris arvicolae* (Grzybek et al. 2015). The prevalence of cestode infection was 20 %, and some of the species reported are for example the most common *Catenotaenia henttoneni* and *Paranoplocephala omphalodes* (Grzybek et al. 2015).

3 MATERIALS & METHODS

3.1 Trapping and sampling

Wild bank voles were captured in Ugglan live traps around two separate trapping locations, Kuhmoinen and Suonenjoki, in Central Finland. Permission for this research was received from the Animal Experimental Board in Finland (license number ESAVI/6935/04.10.07/2016). Voles were euthanized via cervical dislocation. They were then weighted, measured and dissected to collect tissue samples. Parts of lungs, spleen and kidneys were collected in Trisure (Bioline, London, United Kingdom) and stored at -70 °C before RNA isolation. PUUV-specific PCR was performed from the isolated RNA and gastrointestinal helminths were counted in the intestine and categorized into taxa (done by Paula Ahola; personal communication; described in detail in Paula Ahola's upcoming licentiate thesis).

Single cell suspensions from the vole spleens were prepared by passing homogenized fresh spleen through a 70 µM (micromol/liter) cell strainer (Sigma-Aldrich, Saint Louis, United States) with the aid of a syringe plunger. The resulting splenocytes were washed with phosphate buffered saline (PBS) and frozen in CryoStor CS10 freezing medium (Sigma-Aldrich), initially at -70°C for one week and at -135 °C for long term storage.

3.2 Splenocyte stimulation

Splenocyte stimulations were done in a Biosafety Level 3 (BSL3)-laboratory due to the potential presence of Puumala virus in samples. Splenocytes were thawed and 1 millilitres (ml) of growth medium was added. The medium, with a pH of 7,4, contained RPMI 1640 (Thermo Fisher Scientific), 10 % fetal bovine serum (Thermo Fisher Scientific, Waltham, United States), 2 mM l-glutamine, 100 IU/ml of penicillin, 100 µg/ml streptomycin and 25 mM HEPES buffer (Thermo Fisher Scientific). After that, the cells were centrifuged in 800 G-force (g) for three minutes. The supernatant was removed, and 1 ml of growth medium was added. Cells were counted using a Bürker chamber and cells were divided in round-bottomed 96-well plate so that one well had 1 million cells in total in 250 microliters (µl) of growth

medium. Stimulants were applied to individual cells. The stimulants (Table 1) used were general stimulants PHA and phorbol myristate acetate (PMA) with ionomycin, and proinflammatory stimulants lipopolysaccharide (LPS), imiquimod and zymosan. The cells were then grown for three days in a 37 °C incubator with 5 % carbon dioxide (CO_2). After the incubation, cells were centrifuged for three minutes in 800 g. The supernatant was removed, and 400 µl of Tri-reagent (Sigma-Aldrich) was added. The samples were stored in -20°C.

Table 1. Descriptions of cell stimulants used for Th responses of the splenocytes.

Stimulant	Description	Mode of action	Reference
PHA	Lectin derived from a red kidney bean *Phaseolus vulgaris*	T cell mitogen	Martin et al. 2006b
PMA + ionomycin	PMA: a chemical used in research, a tumor promoter, ionomycin: Ca^{2+} ionophore produced by *Streptomyces conglobatus* -bacterium	Activation of protein kinase C, a signal transduction enzyme	Jacquier et al. 2015
Zymosan	A glycan derived from yeast cellular wall	TLR-2 agonist	Dillon et al. 2006
LPS	Endotoxin derived from the cellular wall of Gram negative bacteria	TLR-4 agonist, Th1 response	Hoshino et al. 1999
Imiquimod	Immunomodulatory medicinal compound	TLR-7 agonist, Th1 response	Hemmi et al. 2002

3.3 Extraction of RNA

Both stimulated and unstimulated splenocyte samples were processed the same way from this step on. The samples were thawed, and 80 µl of chloroform was added. The sample tubes were shaken vigorously that the chloroform was mixed properly. The samples were then centrifuged in 12 000 g for 15 minutes. 200 µl of 2-propanol was added to new 1,5 ml Eppendorf tubes. The upper aqueous phase of the centrifuged Tri-reagent (Sigma-Aldrich) tube containing the sample was transferred into new tubes. 1 µl of glycogen (stock 50 mg/ml) was added as an RNA carrier. The samples were incubated in room temperature for 10 minutes. After the incubation, the samples were centrifuged at the speed of 12 000 g for 10 minutes. The supernatant was discarded after that, and 800 µl of 75 % ethanol was added and the tubes vortexed. The samples were stored in -20 ˚C after this when needed before moving to the next step.

The samples were centrifuged for 5 minutes at the speed of 8000 g. The supernatant was discarded. The samples were then air-dried for a minimum of 10 minutes. The RNA pellets were resuspended in 35 µl of sterile diethyl pyrocarbonate (DEPC)-treated water. The solutions were incubated at + 60 ˚C for 5 minutes. If needed, the samples were stored in – 80˚C.

3.4 Reverse transcription

2 µl of the sample RNA was added in 0,5 ml tube containing 1 µl of random hexamer (Thermo Fisher Scientific) and 3,5 µl of DEPC-treated water. The tubes were incubated at +70˚C for 5 minutes. After that, the tubes were directly transferred to cool down on ice, where they were kept for 5 minutes. The reaction mix for reverse transcription (RT) was prepared by mixing 2 µl of 5X RT buffer (Thermo Fisher Scientific), 1 µl of 10 µM dNTP (Thermo Fisher Scientific), and 0,5 µl of reverse transcriptase (RevertAid from Thermo Fisher Scientific) and the mix was added to the tube. The tubes were briefly vortexed to mix the samples. The tubes were incubated with the following program: 25 ˚C for 10 minutes, then 42 ˚C for 60 minutes and then 85˚C for 5 min and then cooled down to 4˚C. The samples were stored in -20˚C if needed.

3.5 Semi-quantitative real-time PCR

PCR reactions were prepared in a 96-well PCR plate. 0,5 µl of the RT-reaction from the previous step was added to the wells containing 6 µl of SYBR Green PCR Master mix (Thermo Fisher Scientific), 5,5 µl of DEPC-treated water and 0,375 µl of gene-specific primers, which were premixed in 10 µM concentration. The genes measured in this study were T-box 21 (*Tbet*) to measure antimicrobial Th1 response, GATA-binding protein 3 (*Gata3*) to measure Th2 response against macroparasites (Jackson et al. 2011), and *β-actin* as a housekeeping gene to normalize the data (Stoltz et al. 2011). The primers for *Gata3* and *Tbet* described by Jackson et al. (2011) and primers for *β-actin* were from Metabion (Steinkirchen, Germany). Real-time PCR was run using AriaMx instrument (Agilent Technologies, Santa Clara, United States).

3.6 Data handling and statistical analysis

The real-time PCR data were analyzed by comparative C_T method (Schmittgen and Livak 2008). C_T refers to threshold cycle and is the number of cycles, after which the fluorescent signal crosses a threshold and is detected (Schmittgen and Livak 2008). The value of C_T is inversely related to the amount of gene expression in the sample (Schmittgen and Livak 2008). The equation for the method is $2^{-\Delta\Delta\ Ct}$ and the value resulted from it is called a fold change (Schmittgen and Livak 2008).

The fold change in the splenocyte stimulation results was counted so that the C_T value of the control gene, β-actin, was first substracted from the C_T values of the studied genes in the same sample. The value of the unstimulated control sample was subtracted from all the stimulated samples from the same individual bank vole. Then the value was exponentiated to a power of two (number two's exponentiation with the value) as shown in the equation. When comparing the unstimulated spleen samples, the median value of all the samples was used as a control value, but otherwise it was done the same way as the stimulations.

The statistical analyses were performed with SPSS software (IBM SPPS version 25, Armonk, United States). Outliers of *Tbet* and *Gata3* expression results were analyzed by the ROUT

method so that all the significant outliers (Q=1%) were removed from the analysis. Kruskal-Wallis H tests were used to compare the effects of the different stimulants to the gene expression of the splenocytes. Kruskal-Wallis tests were performed by comparing the distributions (or mean ranks) of gene expression in stimulant groups, because distributions in the categories were visually inspected to be dissimilar. Mann-Whitney U tests were used to evaluate the potential differences in the gene expression of the splenocytes of PUUV-positive and PUUV-negative bank voles after stimulations. The test was done using distributions (or mean ranks), as the distributions in the groups were different.

General linear mixed models were used to examine the effects of sex, PUUV infection status (presence of PUUV RNA in lungs) and the number of gastrointestinal nematodes on *Tbet* and *Gata3* expression. Trapping location (Suonenjoki or Kuhmoinen) was set as a random factor. All explanatory variables and their interactions were included in the initial models. Stepwise reduction, guided by statistical significance and AIC (Akaike information criterion) value, was then used to determine the most parsimonious final model. Stepwise reductions were done using maximum likelihood, and the final model was done using restricted maximum likelihood. The residuals were checked for normality to verify the models.

4 RESULTS

The study material consisted of 129 trapped wild bank voles. 52 of them were trapped in Suonenjoki and 89 in Kuhmoinen. 51 of the voles were females (39,5%, CL 0,95, 0,32-0,48) and 78 were males (60,5%, CL 0,95, 0,52-0,68). Based on positivity in PUUV RNA-specific PCR in the lung tissue, 59 of the bank voles were found to be PUUV-infected, so PUUV prevalence was 46% (CL 0,95, 0,37-0,54). The number of gastrointestinal nematodes ranged between 1 and 36, with an average of 7 nematodes. Cestodes were found from 7 voles, ranging between 1 to 5 cestodes. All voles infected with cestodes were infected with nematodes as well. No helminths were found from 15 voles, so the helminth infection prevalence was 88 % (CL 0,95, 0,81-0,93).

4.1 Splenocyte stimulations

As expected, *Tbet* expression in stimulated splenocytes (Figure 1), regardless of infection status, had statistically significant differences between different stimulants ($H(4)=29,684$, $p<0,001$). Post Hoc analysis done with pairwise comparisons showed that *Tbet* expression was significantly lower after stimulation with imiquimod than after LPS or PMA-ionomycin stimulations ($p<0,001$). *Tbet* expression was also significantly lower after PHA stimulation than after PMA+ionomycin ($p=0,023$).

All stimulants upregulated *Gata3* levels (Figure 2) but there were no statistically significant differences between different splenocyte stimulations ($H(4)=1,752$, $p=0,781$). Stimulations with imiquimod and PMA+ionomycin caused highest *Gata3* expressions when comparing the mean ranks and inspected visually, but with no statistical significance.

Sample sizes and means of *Tbet* and *Gata3* expression in stimulated splenocytes are shown in Table 2. Means are shown in the table, because the statistical analysis was done using mean ranks of the categories.

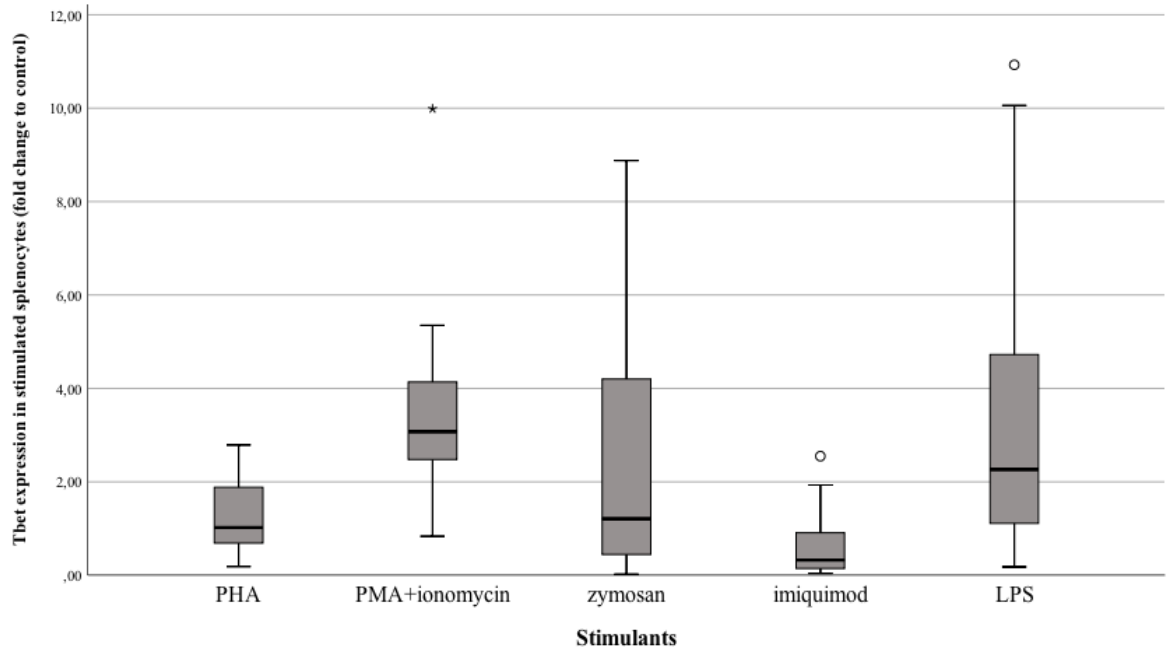

Figure 1. *Tbet* expression (fold change to control sample) in splenocytes after stimulation with PHA, PMA + ionomycin, zymosan, imiquimod and LPS. Differences with statistical significance are between imiquimod and LPS (p<0,001), imiquimod and PMA-ionomycin (p<0,001) and PHA and PMA+ionomycin (p=0,023).

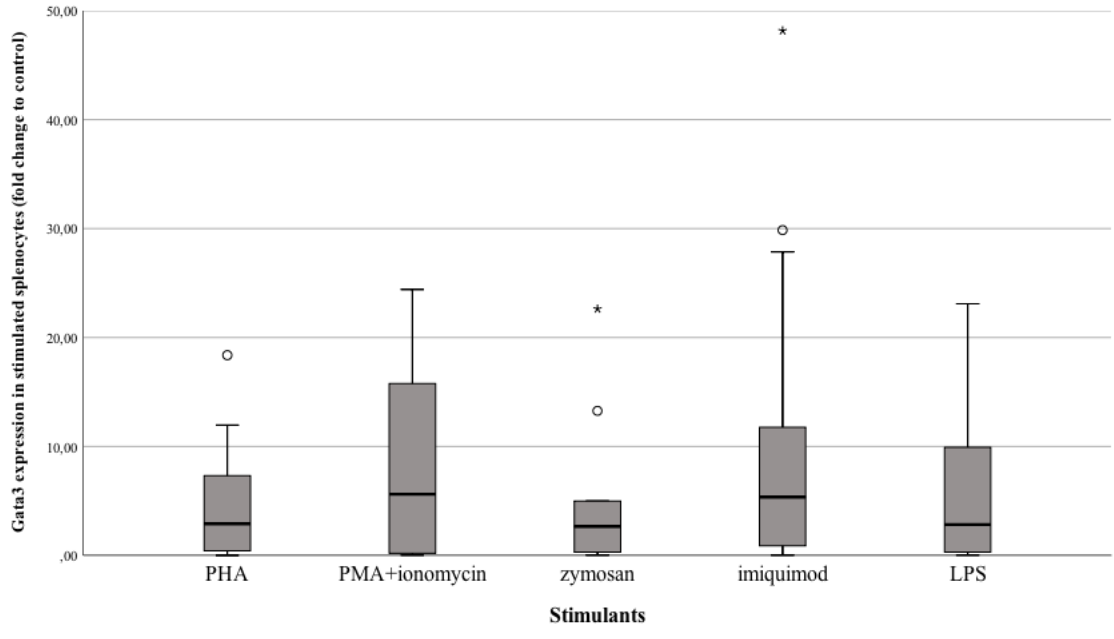

Figure 2. *Gata3* expression (fold change to control sample) in splenocytes after stimulation with PHA, PMA + ionomycin, zymosan, imiquimod and LPS. There are no statistically significant differences between stimulants.

Table 2. Sample sizes (n) and means of *Tbet* and *Gata3* expression (fold change to control sample) after stimulation with PHA, PMA + ionomycin, zymosan, imiquimod and LPS.

Stimulants		*Tbet*	*Gata3*
PHA	n	24	25
	Mean	1,2439	4,4771
PMA+ionomycin	n	11	13
	Mean	3,6754	8,3275
Zymosan	n	12	9
	Mean	2,5892	5,5461
Imiquimod	n	17	19
	Mean	0,6362	10,0058
LPS	n	33	34
	Mean	3,2723	5,7130
Total	n	97	100
	Mean	2,2696	6,5445

Comparing the gene expression levels in the stimulated splenocytes of bank voles infected with PUUV to those without PUUV infection (Figures 3. and 4.), zymosan and PMA+ionomycin had biggest differences between the two groups so that the splenocytes of uninfected bank voles had bigger responses of gene expressions. The difference between two groups was statistically significant after stimulation with PMA+ionomycin with both *Tbet* (U=1,0, z=-2,245, p=0,024) and *Gata3* (U=4,0, z=-2,160, p=0,034) expression. All the other differences were statistically insignificant, including the inspected difference in zymosan stimulation. Sample sizes and means of groups are shown in Table 3 and 4.

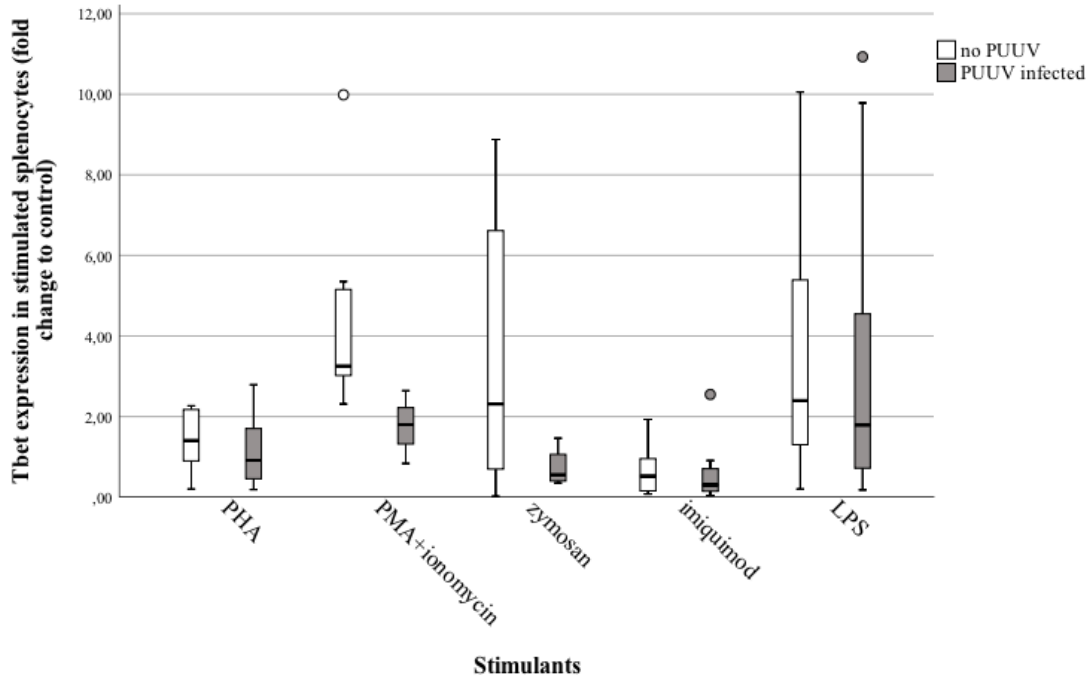

Figure 3. *Tbet* expression (fold change to control sample) in stimulated splenocytes of PUUV-negative and PUUV-positive bank voles after stimulation with PHA, PMA + ionomycin, zymosan, imiquimod and LPS. The difference between PUUV-negative and PUUV-positive voles' samples is statistically significant afer stimulation with PMA+ionomycin (p=0,024).

Table 3. Sample sizes (n) of stimulations and means of *Tbet* expression (fold change to control) in splenocytes after stimulations of PUUV-negative and -positive bank voles and total sample sizes and means.

PUUV status		PHA	PMA+ionomycin	Zymosan	Imiquimod	LPS
No PUUV	Mean	1,4457	4,3941	2,0105	0,6583	3,4272
	n	10	8	6	8	17
PUUV	Mean	1,0999	1,7589	0,7301	0,3749	3,1077
infected	n	14	3	4	8	16
Total	Mean	1,2439	3,6754	1,4984	0,5166	3,2723
	n	24	11	10	16	33

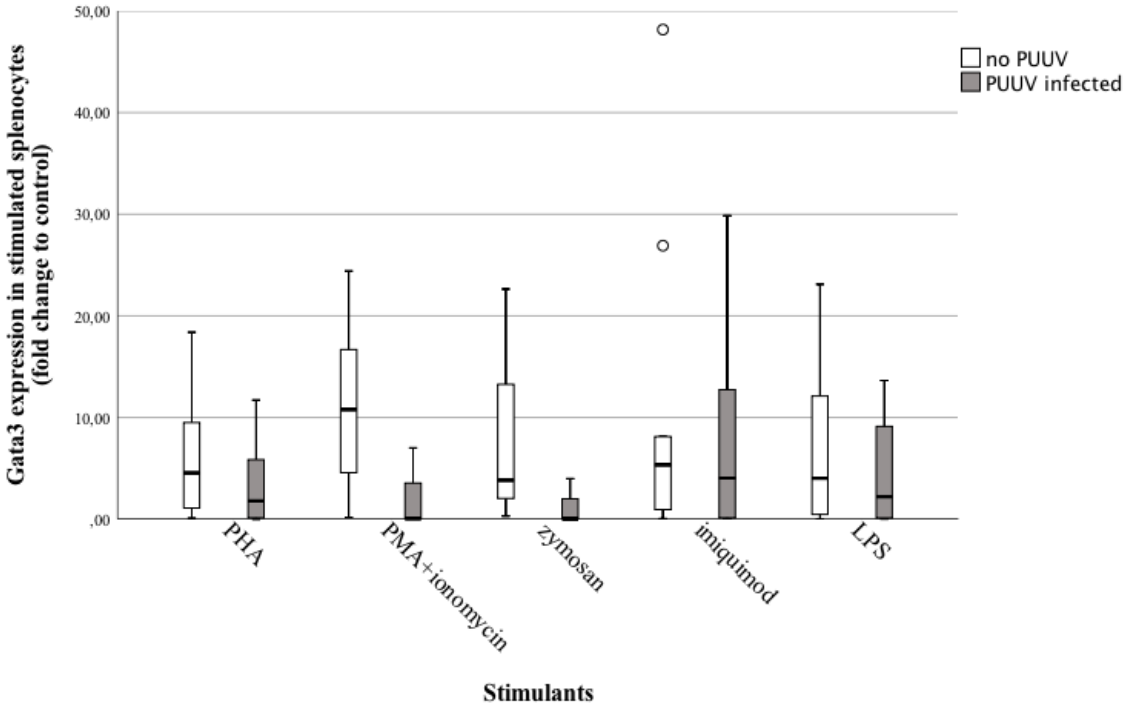

Figure 4. *Gata3* expression (fold change to control sample) in stimulated splenocytes of PUUV negative and PUUV positive bank voles after stimulation with PHA, PMA + ionomycin, zymosan, imiquimod and LPS. The difference between PUUV-negative and PUUV-positive voles' samples is statistically significant afer stimulation with PMA+ionomycin (p=0,034).

Table 4. Sample sizes (n) of stimulations and means of *Gata3* expression (fold change to control) in splenocytes after stimulations of PUUV negative and positive bank voles and total sample sizes and means.

PUUV status		PHA	PMA+ionomycin	Zymosan	Imiquimod	LPS
No PUUV	Mean	4,6938	11,2345	4,6507	6,4097	6,8955
	n	11	9	5	8	20
PUUV	Mean	3,2243	1,7866	1,345	9,0664	4,0236
infected	n	13	4	3	10	14
Total	Mean	3,8978	8,2375	3,4110	7,8856	5,7130
	n	24	13	8	18	34

4.2 *Tbet* and *Gata3* in bank vole spleen

From the sample size n=107, 48% (CL 0,95, 0,39-0,57) of the bank voles were infected with PUUV. 59% (CL 0,95, 0,49-0,68) of the bank voles in this study were males and 41% (CL 0,95, 0,32-0,51) females (Table 5). PUUV prevalence in males was 46% (CL0,95, 0,34-0,58) and in females 50% (CL 0,95, 0,36-0,64).

Table 5. Sample sizes (n) of spleen *Tbet* and *Gata3* expression samples

	PUUV infection	*Tbet*	*Gata3*
Male	No PUUV	34	25
	PUUV-infected	29	17
	Total	63	42
Female	No PUUV	22	20
	PUUV-infected	22	15
	Total	44	35
Total	No PUUV	56	45
	PUUV-infected	51	32
	Total	107	77

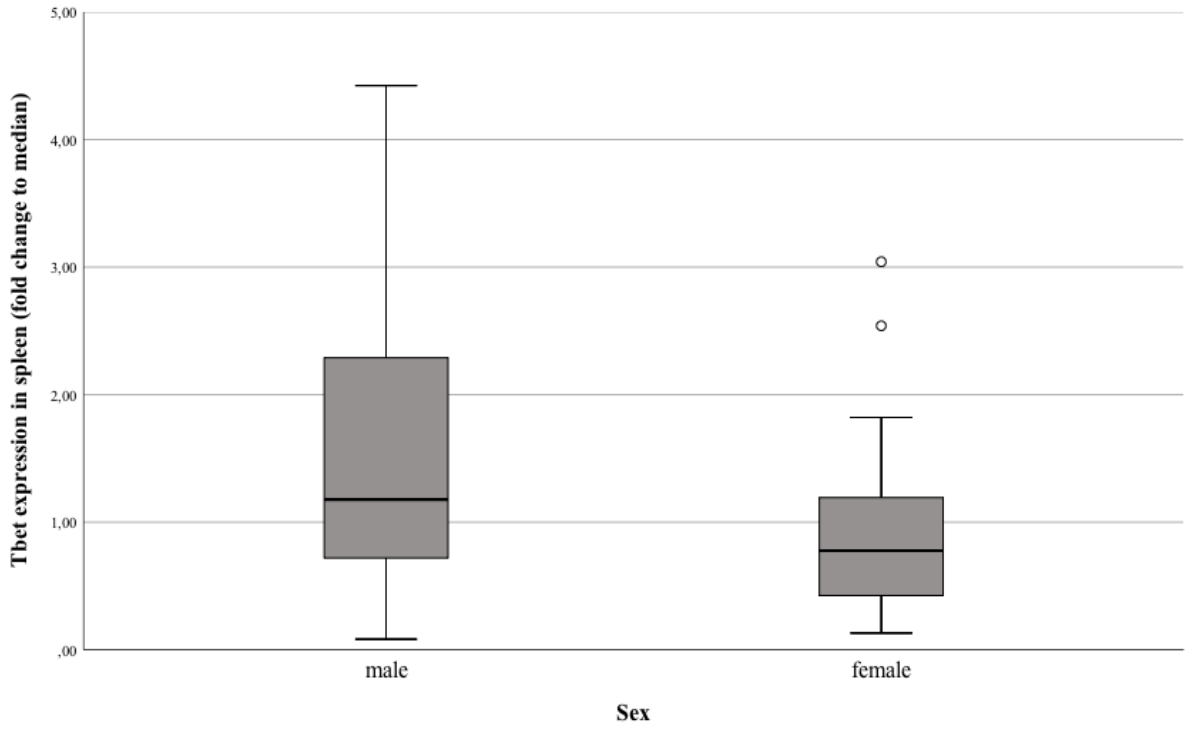

Figure 5. Constitutive *Tbet* expression in spleen (fold change to median) in male and female bank voles (p=0,005).

The constitutive *Tbet* expression in spleen (Figure 5., Table 6.) was higher in males than in females (p=0,005). Other variables in the model (gastrointestinal nematodes and PUUV infection status) didn't have a statistically significant impact on *Tbet* expression (Table 6.).

PUUV infection and gastrointestinal nematode load had a statistically significant (p=0,003) interactive effect on *Gata3* expression of the bank voles so that in PUUV-infected bank voles *Gata3* expression increased with nematode load (Figure 6., Table 6.). In bank voles without PUUV infection, a slight decrease of *Gata3* expression was seen with increasing nematode load. Sex, PUUV infection status and nematode load didn't have a statistically significant effect on *Gata3* expression in spleen (Table 6.).

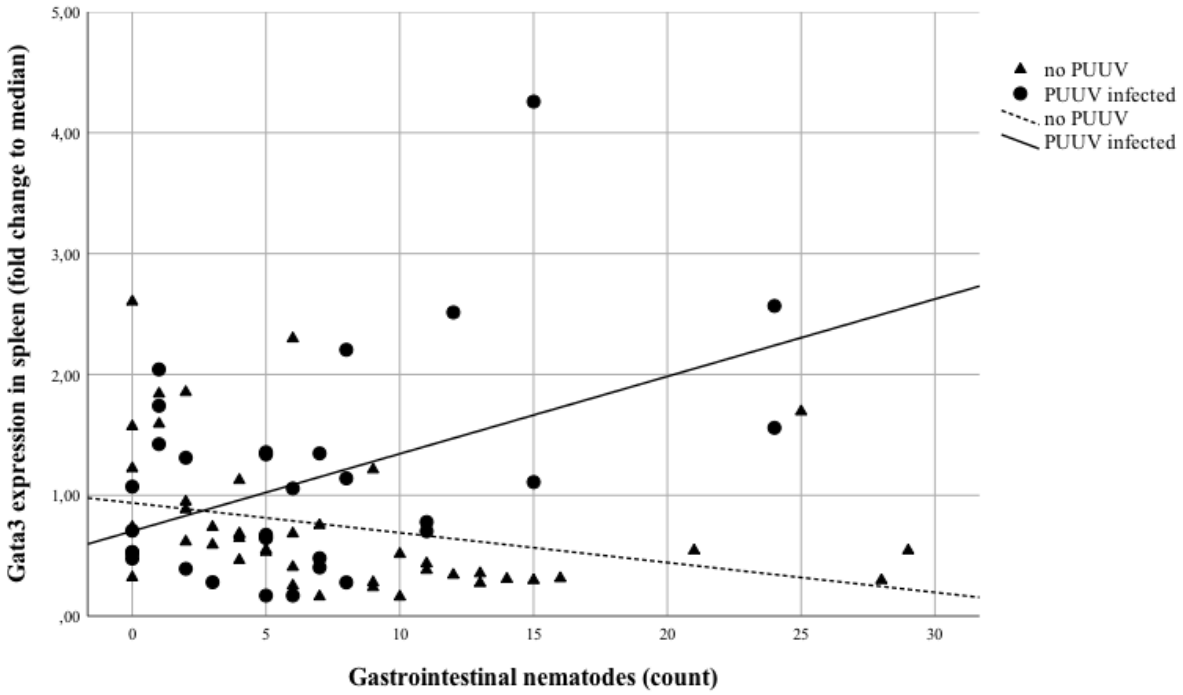

Figure 6. *Gata3* expression in spleen (fold change to median) and the number of gastrointestinal nematodes in PUUV-positive and PUUV-negative bank voles (p=0,003).

Table 6. Result table of general linear mixed model analyses.Tables show the most parsimonious models, the highlighted results are the highest order significant effects on the studied response. Num. d.f=degrees of freedom numerator, Den. d.f.=degrees of freedom denominator, F=F statistic, p=p-value.

Response	Source of variation	Num. d.f	Den. d.f	F	p
Tbet	**Sex**	**1**	**103**	**8,397**	**0,005**
expression	PUUV infection	1	103	1,650	0,202
in spleen	Nematodes	1	103	0,498	0,482
Gata3	Sex	1	71,117	2,356	0,129
expression	PUUV infection	1	71,073	0,563	0,455
in spleen	Nematodes	1	71,319	0,581	0,448
	PUUV infection X Nematodes	**1**	**71,002**	**9,618**	**0,003**

5 DISCUSSION

5.1 Splenocyte stimulations

To investigate the effect of PUUV infection on the ability of bank voles to mount Th responses, I stimulated bank vole splenocytes with various immune stimulants and as a measure of Th1 and Th2 responses, assess the mRNA levels of *Tbet* and *Gata3* after stimulation. However, since the used immune stimulants are thus far better described for model species mice and humans, it was necessary to first investigate their general effects on bank vole Th responses.

5.1.1 General effects of immune stimulants on bank vole Th responses

The pro-inflammatory stimulants LPS, zymosan and imiquimod, were hypothesized to cause a Th1 response with elevated *Tbet* expression, and that was seen with zymosan and LPS, but imiquimod stimulation showed a different pattern causing higher *Gata3* expression. *Gata3* expression did not change much between different stimulants, so pro-inflammatory stimulants activated the Th2 pathway as much as general stimulants.

Imiquimod is a medicine with anti-viral and anti-tumor effects and acts on TLR-7, which induces a production of cytokines of Th1 pathway, TNF-α and IL-12 (Hemmi et al. 2002, Wan and Flavell 2009, Kawai and Akira 2010). Imiquimod stimulation caused a big Gata3 response and much smaller *Tbet* response, which is contradictory to what was hypothesized as it should have induced a Th1 response in splenocytes. The difference between *Gata3* and *Tbet* responses was biggest after imiquimod stimulation and the response it caused was clearly more on Th2 pathway. PUUV infection did not have an effect in the imiquimod stimulation. There could be other factors affecting this, such as helminth load, age or sex, for example.

PMA with ionomycin induces immune cell growth and proliferation and induces Th1, Th2, Th17 and T_{reg} responses by activation of protein kinase C (Jacquier et al. 2015). It also promotes the production of cytokines IL2 and IL4 (Jacquier et al. 2015). PHA is considered as a T cell mitogen, although it is shown to work as a mitogen to multiple other cell types as

well (Martin et al. 2006b). PMA+ionomycin seemed to stimulate the splenocytes more, as both *Gata3* and *Tbet* responses were bigger than after stimulating with PHA.

LPS and zymosan both activate Toll like receptors, LPS on TLR4 and zymosan on TLR6 and TLR2 (Akira et al. 2006). They both induce a Th1 response (Akira et al. 2006). It was seen in this thesis too, LPS and zymosan caused big *Tbet* responses. They both stimulated a *Gata3* response too, although zymosan to lesser extent. The proof of a polarization of Th1 and Th2 responses was seen most clearly with zymosan and slightly with LPS stimulation as well.

5.1.2 Effect of PUUV infection on bank vole Th responses

After stimulations, splenocytes of PUUV-negative bank voles showed stronger responses in almost all categories, although there was a statistically significant difference only after stimulation with PMA + ionomycin.

The apparent suppression of Th cell responses in PUUV-infected voles is intriguing. Whether this is due to a virus specific mechanism or whether there is a vole population defective in T cell functions and thus highly susceptible for PUUV infection, needs further investigation.

Hantaviruses can inhibit the antiviral responses in different cells of host species (Au et al. 2010, Li and Klein 2012), also shown with bank voles and PUUV (Stoltz et al. 2011) and pathogenic hantaviruses can even delay the antiviral response in human, a dead-end host, cells (Kraus et al. 2004, Easterbrook and Klein 2008, Schountz and Prescott 2014). The PUUV-infected voles could have therefore had less effective stimulation response compared to PUUV-negative voles because of the virus inhibiting the proper function of splenocytes. I used stimulants that act on TLRs, which are receptors of antigen presenting cells. Hantavirus SEOV has been shown to inhibit APCs' signaling activity and normal function *in vitro* (Au et al. 2010) so PUUV could act the same way and inhibit the function of the immune stimulants.

Hantavirus infection upregulates the production of T_{reg} cells in its target tissues which leads to downregulation of proinflammatory response (Hannah et al. 2008, Li and Klein 2012). Although it is seen most clearly in the target tissues of the virus and there is evidence that in

spleen, antiviral response is upregulated during acute infection and then returned back to normal (Easterbrook and Klein 2008), it is plausible that immune responses are downregulated in spleen during PUUV infection, especially in the chronic stage. Thus, one explanation to the stimulation results, could be the induction of regulatory T cells by PUUV infection. Helminths can cause immunosuppression and regulatory immune responses in their hosts too (Maizels and Yazdanbakhsh 2003), so helminth infection or helminth and PUUV co-infection could explain the stimulation results as well.

Age correlates with probability of hantavirus infection in hosts in a way that older animals are more likely infected (Khalil et al. 2014) and this has been shown also for bank voles and PUUV (Olsson et al. 2002). Age is shown to change the immune response towards tolerance in bank vole males (Jackson et al. 2014) resulting in aged voles unable to resist the infection efficiently, which could lead to regulatory immune response and immunosuppression and also be one factor explaining the lower stimulation responses in PUUV-infected voles.

The sample size was relatively small in every stimulation category, since splenocyte numbers from several voles were not high enough to perform full stimulation experiments. This could also lead to a bias in the results from spleen sampling, since voles with larger spleens were more likely to be used for stimulation experiments and those could be the most responsive ones to begin with. It would have been interesting to compare the nematode burden, age and sex and other factors' effect on stimulations, but due to small sample sizes, there was not enough statistical power to do that.

Stimulation of splenocytes of wild animals done in the laboratory can be a good way to understand the variation of immune responses as it gives more specific information on the function of immune system as measuring constitutive levels, although it has its limitations. The animals sampled differ so much by their age, sex and reproductive stage, infection history, life-history traits and other physiological and anatomical features, so the sample sizes need to be large to draw any conclusions from the results.

5.2 *Tbet* and *Gata3* in bank vole spleen

In order to investigate the effects of PUUV and nematode co-infections on bank vole Th responses in more rigorous way as compared to splenocyte stimulation assays, I compared the constitutive *Tbet* and *Gata3* mRNA levels in the spleens of bank voles. Due to the increased sample size, I was able to take into account also other factors such as sex and nematode load in the analysis.

Tbet expression was higher in males than in females, which differed from what was expected, as females have been shown to have stronger immune responses and express more genes associated with antiviral responses (Easterbrook and Klein 2008, Hannah et al. 2008). One explanatory factor for lower *Tbet* expression in females can be gestation, as it is shown to cause an immunosuppression and tip the balance of Th1 and Th2 responses on the side of Th2 response (Kidd 2003). *Tbet* expression and Th1 response in bank voles is highly upregulated in the spleen only in the acute stage of PUUV infection, as later on in the course of infection is characterized by a regulatory immune response (Easterbrook and Klein 2008). *Tbe*t expression would thus be best interpreted together with more accurate PUUV infection status details, such as measuring antibodies and viral load.

The prevalence of PUUV was almost the same in both groups, whereas in other studies the prevalence of hantaviruses have been higher in males (Easterbrook and Klein 2008, Jackson et al. 2014). The prevalence of PUUV in older males was higher than in older females outside the population peak years in a study of Escutenaire et al. (2002), so the stage of the fluctuating population could have had an effect to these results. During the peak years, PUUV prevalence could be more balanced between males and females. Reproduction status and age probably influence these results, but those were not accounted for in this thesis.

Gata3 expression level correlated with nematode burden in PUUV-infected bank voles, which was not seen with PUUV-negative bank voles. Jackson et al. (2014) showed that *Gata3* was higher in older field voles with nematode burden and linked it to infection tolerance. *Gata3* was also linked to improved body condition and improved survival (Jackson et al. 2014). In younger males, the immune response was more resistive (Jackson et al. 2014). One explanation for these results could then be, that the bank voles with no PUUV infection could

have been younger, as age has been shown to correlate with PUUV infection (Olsson et al. 2002, Khalil et al. 2014), with less tolerant immune response against helminths and thus weaker *Gata3* expression. Comparing the age groups would be interesting and might give more information on this.

Gata3 expression could be weaker in PUUV-negative bank voles because of high upregulation of antiviral Th1 response resisting PUUV infection and changing the balance towards Th1 pathway, but then there should have also been a difference in *Tbet* expression between PUUV-negative and PUUV-positive bank voles, which was not seen in these results.

Gata3 has been recognized as a transcription factor of a regulatory T cell response as well as Th2 pathway (Wang et al. 2011). PUUV infection causes regulatory immune response in its host species (Easterbrook and Klein 2008). Helminths can suppress the immune system of their hosts (Maizels and Yazdanbakhsh 2003) and host response to helminth infection is characterized by regulatory T cells and immunoregulatory cytokines (Anthony et al. 2007, McSorley and Maizels 2012). The explanation for the effect of the interaction of nematode load and PUUV infection on *Gata3* expression can be a combination of a chronic nematode infection and a chronic PUUV infection of its reservoir host. These results can thus be considered as further proof of *Gata3* being a marker for tolerance response against parasites and consistent with the results of Jackson et al. (2014).

In this study, the assays used are designed to look at bulk immune responses instead of pathogen-specific ones. That is why the assays pick up the "super responders", and with a big *Gata3* response, the bank voles could have been less capable of mounting a Th1 response against virus infections because of the trade-off between Th1 and Th2 responses and become infected with PUUV more easily. To confirm the Th1/Th2 trade-off hypothesis still needs more research though in natural populations. There was a lot of very high *Gata3* expression results, which needed to be removed from the data as outliers, so *Gata3* response seems to be an important part of immune responses in bank voles.

Measuring the constitutive levels of immunological markers in wild animals is a good way to gather information on them, but are hard to interpret, because it is impossible to know their infection history and all the other physiological and environmental factors that influence the immune responses. In this study, only PUUV and intestinal helminths were accounted for, but

the bank voles were probably infected with other pathogens, such as other viruses or bacteria, as well, which could have influenced the results. With a bigger sample size, also the influence of age, reproduction status and body condition could have been looked at as well and should be studied in the future.

All in all, more research is needed on wild animals in natural populations to understand co-infections and immune responses and how natural conditions affect them. Combining laboratory research and field studies is most likely the best way to get information and understand the causes and drivers of variation in natural populations.

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